Increased amount of type III pN-collagen in human abdominal aortic aneurysms: Evidence for impaired type III collagen fibrillogenesis

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Purpose: **This study aimed to characterize the distribution of structural domains of type I and III collagens in the wall of abdominal aortic aneurysms (AAAs), by the use of undilated atherosclerotic aortas (aortoiliac occlusive disease [AOD]) and healthy abdominal aortas as controls.**
Methods: **Immunohistochemical staining was applied with antibodies for the aminoterminal propeptides of type I (PINP) and type III (PIIINP) procollagens, which represent newly synthesized type I and III pN-collagens. In addition, an antibody against the aminoterminal telopeptide of type III collagen (IIINTP) was used as a means of detecting maturely cross-linked type III collagen fibrils.**
Results: **The newly synthesized type III procollagen detected by means of PIIINP staining was concentrated in the media in aneurysmal aortas, whereas type I pN-collagen was localized in the intima in both AAAs and AODs. The healthy aortas showed no immunoreactivity for either PIIINP or PINP. The cross-linked type III collagen, detected by means of IIINTP staining, stained transmurally in all study groups, but appeared more abundant in the media in AAAs.**
Conclusion: **Our results strongly suggest that the metabolism of type III collagen is enhanced in AAAs. Intensive type III pN-collagen staining was present mainly in the media layer in AAAs, suggesting a role of type III collagen in aneurysm formation, whereas type I pN-collagen was present in the intima in both AAAs and AODs, suggesting that type I collagen synthesis is a fibroproliferative response related to the atherosclerotic process. The increased type III pN-collagen in AAAs may result in impaired fibril formation and, thus, in decreased tensile strength of aneurysmal tissue. (J Vasc Surg 2000;32:1201-7.)**

Aneurysm formation is a complex remodeling process that involves both synthesis and degradation of the proteins of the extracellular matrix.[1,2] Abdominal aortic aneurysms (AAAs) are characterized by structural deterioration of the aortic wall leading to progressive aortic dilatation and, eventually, rupture. The two major structural elements of the aortic wall are collagens and elastin, the structural properties of which are regarded as mutually complementary. Elastin provides the aortic wall with distensibility, whereas collagens are responsible for its tensile strength. Therefore, it has been suggested that the degradation of arterial elastin seen in aneurysms leads to vessel dilatation and decreased distensibility, whereas the degradation of fibrillar collagens results in decreased tensile strength and vessel rupture. Thus, the critical element in both the enlargement and the rupture of the aneurysms may reside in collagens.[3,4]

The fibrillar collagen network of the abdominal aorta consists predominantly of type I and III collagens, the extensile characteristics being attributed to

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Competition of interest: nil.
Supported in part by grants from National Technology Agency (Tekes), Finland, and the Oulu University Hospital.
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0741-5214/2000/$12.00 + 0 **24/1/109743**
doi:10.1067/mva.2000.109743

type III collagen.[5] Collagens are continually synthesized throughout life, and the collagen content in the vessel wall thus reflects the net effects of synthesis and degradation.

Type I and III collagens are synthesized and secreted into the extracellular space as procollagens, with large extra parts known as propeptides at both ends.[6] The changes in collagen turnover can be followed easily by measuring the concentrations of circulating procollagen propeptides.[7] We have previously shown that the metabolism of type III collagen is accelerated in AAA tissue, a conclusion based on measuring the aminoterminal propeptide of type III procollagen (PIIINP) in blood.[8,9]

In the current study, we used specific antibodies against different structural domains of the main aortic fibrillar collagens. The stainings for the aminoterminal propeptides, PINP and PIIINP, represent the newly synthesized type I and type III pN-collagens, respectively, with these propeptides still attached. Between the propeptides and the helical collagen molecule, there are short non–triple-helical telopeptides containing the sites for intermolecular cross-linking. A structure called IIINTP represents the aminoterminal cross-linked telopeptide of type III collagen, and, thus, the third antibody, the one against IIINTP, recognizes old, already cross-linked and fully matured type III collagen fibers. We characterized the distribution of these structural domains of collagens in the walls of AAAs, in aortoiliac occlusive disease (AOD), and in healthy aortas.

PATIENTS AND METHODS

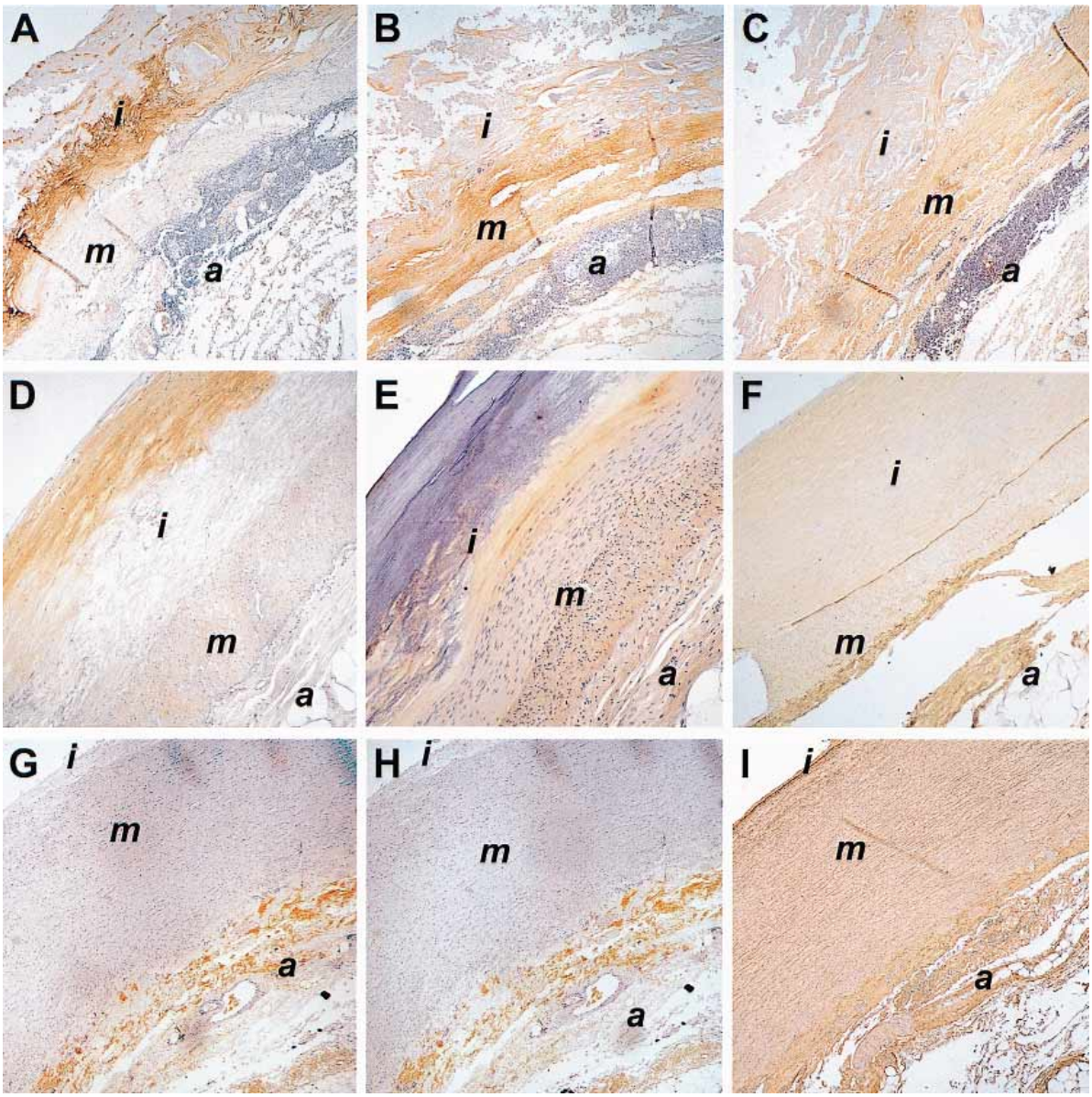
Full-thickness longitudinal strips of the anterior wall of the infrarenal aorta were obtained from 19 consecutive patients (15 men and 4 women; mean age, 69 years; range, 59-79 years) who underwent elective aortic reconstruction for degenerative AAAs at the Department of Surgery, Oulu University Hospital. All the aneurysms were degenerative and fusiform, and their maximum diameters had been measured preoperatively by means of ultrasound scan (mean diameter, 5.2 cm; range, 4.3-6.4 cm). The aneurysm specimens were compared with those from the abdominal aorta of nine cadavers (6 men and 3 women; mean age, 54 years; range, 40-71 years) with AOD. Among the general demographic data, there were no significant differences between the two groups (AAA vs AOD: diabetes mellitus, 5 of 19 vs 3 of 9; coronary artery disease, 11 of 19 vs 6 of 9; chronic obstructive pulmonary disease, 8 of 19 vs 4 of 9; hypertension, 10 of 19 vs 5 of 9). Two control specimens were obtained from the abdominal aortas of two cadavers (16 and 61 years). The control subjects had no symptomatic vascular disease, and this information was supported by the absence of significant pathologic vascular changes at autopsy. All autopsies were performed within 24 hours of death. All specimens were formalin-fixed and paraffin-embedded. The collection of tissue samples was approved by the local ethics committee.

Monospecific, polyclonal antibodies against the aminoterminal propeptides of type I (PINP) and type III (PIIINP) procollagens and the aminoterminal telopeptide of type III collagen (IIINTP) were used. The PINP[10] and PIIINP[11] antigens were purified from the ascitic fluid of patients with malignant tumors, and the IIINTP antigen[12] was purified from human uterine leiomyoma. The antisera were raised in rabbits and purified by means of immunoaffinity chromatography with cross-adsorption on several columns with the possible cross-reacting human extracellular matrix antigens (PINP, PIIINP, IIINTP, ICTP, 7S domain of type IV collagen, and/or P1 domain of laminin) and, finally, with the antigen (PINP, PIIINP, or IIINTP) used in the immunization coupled to CNBr-activated Sepharose 4B. The lack of cross-reaction was confirmed by showing that the antibodies did not bind any of several radioactively labeled test antigens (eg, no binding of PIIINP-I^{125} by the anti-PINP antibodies).

The avidin-biotin-peroxidase method was used for the immunostainings. Sections 5 μm thick were deparaffinized and incubated in a solution containing 0.14 g trypsin and 0.1 g $CaCl_2$ in 100 mL of 0.2 mol/L ammonium bicarbonate, pH 7.8, for 40 minutes at 37°C. After the trypsin pretreatment, the slides were washed in phosphate-buffered saline, and the specific rabbit antihuman antibodies (dilution 1:50 for anti-IIINTP, 1:10 for anti-PIIINP, and 1:100 for anti-PINP) were applied on the slides, followed by the biotinylated secondary antirabbit antibody and the avidin-biotin-peroxidase complex. The color reaction was developed with diaminobenzidine. All samples were trypsinized and stained at the same time. In the control stainings, phosphate-buffered saline was substituted for the primary antibody.

To identify smooth muscle cells in the medial layer of the aortic wall in aneurysms, we used commercially available antibodies against smooth muscle α-actin (Clone 1A4, Sigma Biosciences, St Louis, Mo) at a dilution of 1 to 1000. The maximal and minimal diameters of the positively stained layer were measured with an ocular micrometer.

The collagen staining reactions were evaluated



Immunohistochemical type I and III collagen staining of abdominal aortic aneurysm (**A, B,** and **C**), atherosclerotic abdominal aorta (**D, E,** and **F**), and healthy abdominal aorta (**G, H,** and **I**). **A, D,** and **G**, Staining of type I pN-collagen; **B, E,** and **H**, staining of type III pN-collagen; and **C, F,** and **I,** staining of cross-linked type III collagen (original magnification, 10×; hematoxylin counterstain). The *brown color* in **G** and **H** is blood, not collagen staining.

separately by two pathologists (Y.S. and J.M.) in a blinded fashion. There was a statistically significant association between the evaluations of these two observers (P = .038, Fisher exact test). The immunostainings for the different collagen domains were assessed separately in the intimal, medial, and adventitial layers in all samples whenever possible. The staining reactions were semiquantitatively classified into 4 groups: –, no staining; +, weak staining; ++, moderate staining; and +++, strong staining. The Fisher exact test was used for statistical analyses; in the statistical calculations, the staining intensities were treated as two groups: –, + and ++, +++. In Table IV, we reduced the staining intensity data into two groups (A and B) to ease the comparisons between AAA and AOD.

Table I. Intensities of PINP staining in different layers of AAA

	*PINP**		
Staining intensity	*Intima*	*Media*	*Adventitia*
No	0	12	11
Weak	0	5	4
Moderate	5	0	1
Strong	12	0	0

*Number of specimens showing the given staining intensity. In some cases, it was not possible to evaluate the particular layer. Statistical significance between the layers was calculated by means of the Fisher exact test: intima versus media, $P = .0001$; intima versus adventitia, $P = .0001$; media versus adventitia, $P = .48$.

Table II. Intensities of PIIINP staining in different layers of AAA

	*PIIINP**		
Staining intensity	*Intima*	*Media*	*Adventitia*
No	1	0	2
Weak	5	1	6
Moderate	4	0	2
Strong	9	17	7

*Number of specimens showing the given staining intensity. In some cases, it was not possible to evaluate the particular layer. Statistical significance between the layers was calculated by means of the Fisher exact test: intima versus media, $P = .05$; intima versus adventitia, $P = .27$; media versus adventitia, $P = .007$.

RESULTS

Type I and III procollagen antigens are present in the diseased aortic wall. The PINP, PIIINP, and IIINTP antigens were all detected with variable intensity in the diseased aortas, whereas the healthy aortas showed staining only for IIINTP. This is understandable, because the aminoterminal propeptides detected by means of anti-PINP and anti-PIIINP are present on newly synthesized type I and III collagen fibers, respectively, whereas IIINTP represents the mature, cross-linked structure of type III collagen. Thus, increased metabolic activity can be detected by means of the staining intensity of PINP and PIIINP.

The average diameter (± SD) of the medial layer as determined by means of the immunostaining for smooth muscle α-actin in the aneurysmal walls was 365 ± 227 μm, whereas in the atherosclerotic lesions it was 796 ± 446 μm ($P = .004$). In the healthy aortic samples, it was 1200 to 1400 μm.

Abdominal aortic aneurysms. A very strong linear reaction was seen with the anti-PINP in the intimal layer of AAAs, whereas such staining was only slight or completely absent in the medial and adventitial layers (Table I; Figure, *A*). For PIIINP, the strongest linear immunostaining was seen in the medial layer (Figure, *B*). The reaction was weaker in the intimal layer ($P = .052$) and significantly weaker in the adventitial layer ($P = .007$; Table II). IIINTP reactivity was seen as strong diffuse positivity in the adventitial and the medial layers of AAAs. A significant difference between the intimal and medial layers ($P = .02$; Table III; Figure, *C*) was revealed by means of the immunoreactivity.

The differences in immunoreactivity between the AAA and AOD groups are summarized in Table IV. All the layers of the aneurysmatic aortic wall showed stronger staining for PIIINP than did the corresponding areas in the AODs (Table IV). The signal for PINP, which was very strong in the intimal layer of AAAs, was only slight or absent in the media. A similar trend was seen in the atherosclerotic specimens, although the intimal staining was somewhat weaker (Table IV). For IIINTP, the difference was only evident in the medial layer, which again showed more intense IIINTP staining in the AAAs (Table IV).

Aortoiliac occlusive disease and healthy aorta. Weak to strong (+ to +++) positivity with anti-PINP was seen in the intimal layer of the atherosclerotic undilated aorta (Figure, *D*). Thus, there was a significant difference in the AOD specimens between the intima and both the media and the adventitia ($P = .03$). The PIIINP staining (Figure, *E*) was weak (– to +) and the IIINTP staining (Figure, *F*) was moderate (+,++) transmurally, and no statistically significant differences in either PIIINP or IIINTP

Table III. Intensities of IIINTP staining in different layers of AAA

	*IIINTP**		
Staining intensity	*Intima*	*Media*	*Adventitia*
No	1	0	0
Weak	5	0	2
Moderate	3	2	3
Strong	10	16	12

*Number of specimens showing the given staining intensity. In some cases, it was not possible to evaluate the particular layer. Statistical significance between the layers was calculated by means of the Fisher exact test: intima versus media, $P = .02$; intima versus adventitia, $P = .15$; media versus adventitia, $P = .23$.

Table IV. Staining intensities of PINP, PIIINP, and IIINTP in the layers of the aortic wall, and their differences between AAAs and AOD

	*PINP**		*PIIINP**		*IIINTP**	
	AOD	*AAA*	*AOD*	*AAA*	*AOD*	*AAA*
Intima						
A	3	0	8	6	5	6
B	4	17	1	13	4	13
P value	.017†		.006†		.2	
Media						
A	7	17	7	1	6	0
B	0	0	0	17	1	18
P value	1		.00009†		.00004†	
Adventitia						
A	7	15	6	8	3	2
B	0	1	0	9	3	15
P value	.7		.03†		.09	

*Number of specimens showing the given staining intensity. In some cases, it was not possible to evaluate the particular layer.
†Statistically significant *P* values.
A, No or weak immunoreactivity; *B,* moderate or strong immunoreactivity.

immunoreactivity between the different layers of the atherosclerotic aortic wall were detected.

The samples of healthy aorta revealed no immunoreactivity for either PINP (Figure, *G*) or PIIINP (Figure, *H*). There was, however, constant transmural IIINTP staining (Figure, *I*).

DISCUSSION

In the pathogenesis of AAA, the fragmentation and decrease of elastin in the medial layer[13] shift the biomechanical stress in the aortic wall to other structural elements, collagens in particular. Thus, type III collagen, with its extensile properties,[5] may play a role in resisting pulse pressure. The current study gives further support to a role of type III collagen in AAA pathogenesis, because newly synthesized and incompletely processed type III pN-collagen was revealed in the media of the aneurysmal aorta. The collagen content in AAAs may be maintained or even increased because of the enhanced rate of collagen synthesis.[14,15] However, this new collagen may have delayed cross-link maturation.

The structures recognized in the tissue by means of anti-PINP or anti-PIIINP antibodies are collagen fibers on the surface of which the type I or type III, respectively, collagen molecules still have the aminoterminal propeptides uncleaved. Because of the repeated washings and the trypsin treatment, the corresponding free propeptides are not likely to be present in the final tissue specimens.

Newly synthesized type III pN-collagen was mainly concentrated in the media in the aneurysmal aortas. The type III pN-collagen is more susceptible to proteolysis, because the cross-link formation at the aminoterminus is delayed because of the presence of the bulky propeptide domain. This is important, because there are several reports on the increased amount of collagen-degrading matrix met-

alloproteinases (MMPs)[16-18] and cathepsins[19] in AAA. Interesting new data suggest that the distributions of MMPs in atherosclerotic and aneurysmal aortas are different. In AODs, MMPs have been found on the intimal side of the tunica media, whereas their preferential location in AAAs is within the adventitia and on the adventitial side of the media.[20] Thus, the type III collagen in the media of an aneurysm might be under a more severe proteolytic attack than the corresponding area in an atherosclerotic aorta without aneurysmal changes. Furthermore, the PIIINP remaining attached to the collagen molecule has the capacity to regulate fibril diameter.[21] Therefore, increased amounts of type III pN-collagen may leave the fibers thinner and thus lead to a decrease in tensile strength and to the formation of an AAA. Unfortunately, there is not much data about the structure and regulation of the type III procollagen N-proteinase.[22] The marked increase of PINP-staining in the intima in AAA samples indicates active type I collagen synthesis, probably caused by the atherosclerotic injury often associated with AAAs. This is supported by the similar finding of PINP immunoreactivity in the AOD samples. An increase in the rate of type I and type III collagen synthesis has been found (eg, in ovarian carcinoma[23]), although the collagen content of the tissue is decreased, together with incomplete maturation of collagen cross-linking.[24]

Accordingly, the PIIINP and IIINTP immunostainings together suggest that the type III collagen was present in the adventitia in its mature, fully processed form. None of the aneurysms studied had ruptured. Thus, the enhanced collagen turnover in the media, which was seen as increased staining of type III pN-collagen, could represent the initial or midterm dilatation process. However, the adventitial extracellular matrix of the aneurysm must also fail to enable rupture. After removal of the media, the aortic adventitia seems capable of maintaining the normal aortic diameter throughout the range of expected blood pressures.[25] For example, aortic endarterectomy, in which the intima and part of the media are removed, does not cause aneurysm formation. In a healthy person, even the adventitia may provide enough mechanical support to prevent dilatation, but under predisposing circumstances, an aneurysm would develop. The AAA-related alterations are perhaps first manifested in the medial layer, and these phenomena serve as a window for further observations about the progression of the dilatation seen clinically. Our previous investigations into the evident increase of PIIINP in patient serum[8,9] together with the current study suggest that the PIIINP antigen also found in blood reflects the metabolic situation in AAA tissue and thus strengthens the idea of the usefulness of the PIIINP assay as an indicator of the aneurysmatic process in clinical practice.

The AODs were positive for IIINTP but not for PIIINP, indicating that type III collagen undergoes full processing into its mature, cross-linked forms and that the half-life of type III collagen may be longer and its turnover slower than that of AAA. This finding and the nonexistent differences between the layers of the aortic wall in AOD samples are consistent with the idea of metabolic inertia in silent atherosclerotic lesions, such as in carotid plaques, in which we have previously found the type I and III collagens to be fully cross-linked and the amount of type III pN-collagen to be negligible.[12] Thus, it seems that there is enough time in atherosclerosis for complete collagen maturation and processing, whereas in AAAs the demand for repair exceeds the proper processing of collagen molecules. Not only the increased amount of type III pN-collagen, but also the defects in the synthesized collagen might result in impaired fibril formation, because mutations in the type III collagen gene have been found in patients with a familial tendency to AAAs.[26,27]

Our results are in accordance with the idea that the synthesis of type III collagen is enhanced in AAAs. Furthermore, the newly synthesized type III collagen was present mainly in the medial layer, suggesting impaired mechanical support and emphasizing the significant role of type III collagen in AAAs. Type I pN-collagen, in turn, was only present in the intima, where the atherosclerotic lesion develops.

We thank Ms Liisa Kaarela, Ms Mirja Vahera, and Ms Eija Tomperi for their expert technical assistance.

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Submitted Feb 7, 2000; accepted May 16, 2000.